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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Bisgaard-Frantzen et al. Confirmation No: 8501

Serial No.: 10/025,648 Group Art Unit: 1751

Filed: December 19, 2001 Examiner: Prouty

For: Amylase Variants

DECLARTION OF TORBEN V. BORCHERT UNDER 37 C.F.R. 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Torben V. Borchert, do hereby declare as follows:

1. I am a Director at Novozymes in Bagsvaerd, Denmark, where I am responsible for research and development in protein design. I have been an employee at Novozymes since August 1993. I have held positions as research scientist, senior scientist, manager and director and have been involved in and directed work on improving the properties of industrial enzymes by protein engineering techniques for the complete period. Various enzymes have been addressed including alpha-amylases. Details of my education, professional experience and publications are included in the copy of my *Curriculum Vitae* which is attached as an Appendix hereto.

2. I am an inventor of the subject matter claimed in U.S. Application Serial No. 10/025,648. I am familiar with Suzuki et al.. J. Bio. Chem, Vol. 264, No. 32, 18933-18938 (1989) and Bisgaard-Frantzen et a. WO 95/10603.

3. Under my supervision, an experiment was carried out comparing the deletion of R179-G180 in *Bacillus stearothermophilus* alpha-amylase (BSG) to the deletion of R176-G177 in *Bacillus amyloliquefaciens* alpha-amyase (BAN), as described below.

4. A BSG variant having the deletion of R179-G180 (BSGdel) and a BAN variant having the deletion R176-G177 (BANdel) were constructed by standard mutagenesis methods and the sequences verified by DNA sequencing. Other than the deletion of R179-G180 in BSG and the deletion of R176-G177 in BAN, the variants were otherwise identical to the wild type alpha-amylases.

5. *Bacillus subtilis* strains expressing the wild type enzyme or variant enzyme, respectively for BAN and BSG, were grown under identical conditions in PS-1 media in shakeflasks for 4 days at 37 °C at 275 RPM, and were harvested by centrifugation of the samples for 5 minutes at 20,000 RMP, thus separating the cell pellet and the supernatant. The amylase containing supernatants were tested for residual activity after thermal inactivation carried out at 80 degree Celsius in a Britton Robinson (B-R) Buffer, pH: 5.9, and half life was calculated. The temperature of 80 degree Celsius was chosen as the highest temperature where both BAN and BSG wild type and derived variants could be reliably compared. The supernatants were diluted in B-R buffer to a suitable activity level and were aliquoted in 8 portions of 100 µl each. These samples were heat-treated in a PCR machine for the indicated times and at the indicated temperatures. The heat treatment was stopped by transferring the samples to ice and the samples were left there until activity was measured and residual activity calculated.

Experimental protocol:

1) Centrifuged the sample for 5 minutes at 20.000 rpm. Use the supernatant.
2) Dilute the sample in BR buffer pH 5.9 in order to achieve an activity level that will result in an OD650 of approximately 1.0 in the un-heated sample.
3) Substrate : Suspend 1 Phadebas amylase test tablet from Pharmacia in 5 ml B-R buffer pH 5.9.
4) 575 µl substrate in 1.5 ml eppendorftube is preheated 5 min. at 37 °C with shaking.
5) Add 25 µl sample at time 0 and continue shaking.
6) Add 100µl 1 M NaOH at time 15 min to stop the reaction.
7) Centrifuge the samples 5 min 20.000rpm.
8) Pipet 200 µl supernatant in a microtiterplate
9) Measure end-point at 650 nm.

Buffers:

B-R (Britton Robinson) buffer pH 5.9

50 mM acetic acid, 50 mM boric acid, 50 mM phosphoric acid,0.1 mM calcium chloride and 0.01% BRIJ 35 are mixed and pH is adjusted to 5.9 with NaOH.

6. Thermal inactivation trials. For BAN wild type and BAN variant one experiment was carried out with double determination for each time point. For BSG wild type and BSG variant two series of experiments were carried out due to the necessary, long incubation times.

Residual activity data (100% equals zero time) from the experiment is shown below.

BAN Wild type:

	Min	Res act
1	0.0	102.697998
2	0.0	97.302002
3	0.5	96.083551
4	0.5	97.127937
5	1.0	57.789382
6	1.0	64.055701
7	1.5	55.526545
8	1.5	50.826806
9	2.0	40.557006
10	2.0	43.342037
11	3.0	25.587467
12	3.0	27.850305
13	4.0	2.959095
14	4.0	2.785030

BANdel

	Min	Res act
1	0	100.108814
2	0	99.891186
3	5	78.781284
4	5	80.087051
5	10	48.095756
6	10	49.183896
7	20	25.244831
8	20	32.861806
9	40	5.223069
10	40	5.875952

BSG Wild type

	Min	Res act	exp
1	0	99.19817	1
2	0	100.80183	1
3	20	89.57617	1
4	20	90.49255	1
5	40	77.09049	1
6	40	76.51775	1
7	60	68.72852	1
8	60	71.82131	1
9	90	60.71019	1
10	90	61.28293	1
11	120	44.10080	1
12	120	47.99542	1
13	0	99.37947	2
14	0	100.62053	2
15	20	90.31026	2
16	40	85.91885	2
17	65	74.84487	2
18	65	78.37709	2

BSGdel

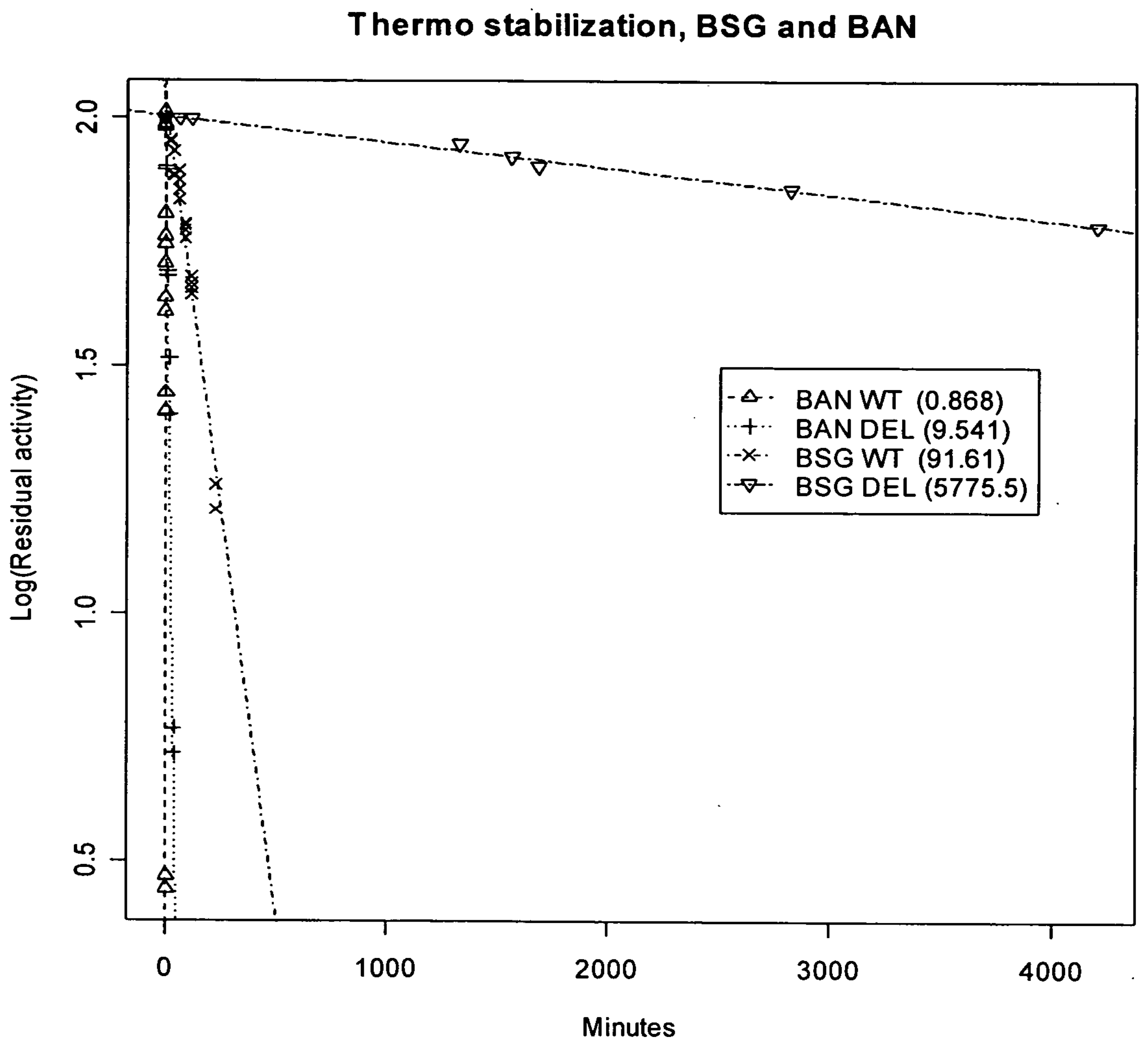
	min	res act	exp
1	0	100	1
2	65	100	1
3	120	100	1
4	1320	89	1
5	1560	84	2
6	1680	80	1
7	2820	72	2
9	4200	61	2

		19 90 57.37470 2 20 90 59.37947 2 21 120 46.58711 2 22 120 45.53699 2 23 225 16.22912 2 24 225 18.23389 2	

7. For each data series, a regression line was computed and the half-life was computed based on the regression line. The two data-series for BSG wild type gave different slopes (p=0.01), so they were treated both separately and as one series. The two data series for BSGdel give consistent slopes (p=0.96), so they are treated as one series. In the table, the half-lives are compared and the improvement factors are compared. The numbers in parenthesis corresponds to the two data series on BSG wild type treated separately.

	Half-life @ 80 degree Celcius	Improvement	Relative improvement
BAN WT	0.9 min		
BANdel	9.5 min	11x	
BSG WT	92 min (87-111)		
BSGdel	5775 min	63x (52x – 66x)	5.7x (4.7x – 6x)

8. A graphical illustration is provided below.



9. The deletion of R179-G180 in BSG has a pronounced and very surprising effect on the thermal stability in BSG as compared to the deletion of R176-G177 in BAN. The deletion of R179-G180 in BSG causes a 63 fold increase in half-life at 80 degree Celsius whereas the deletion of R176-G177 in BAN causes only an 11 fold increase in half-life at the same conditions. The deletion of R179-G180 in BSG gives a relative improvement of thermal stability which is 5 to 6 times higher than what is seen in BAN having the deletion of R176-G177. These results are statistically significant and very surprising as the effect of the double deletion in BSG is significantly greater than what would have been expected based on the

combined teachings of Suzuki et al. (JBC 260:6518, 1989) in view of Bisgaard-Frantzen et al., WO 95/10603. The statistical analysis is attached as Appendix 1.

10. All statements made herein of my own knowledge are true and all statements made herein on information and belief are believed true. Further, I am aware that willful false statements and the like are punishable by fine, imprisonment, or both, 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the involved Svendsen application, as well as the position of Novozymes in the above-captioned interference.

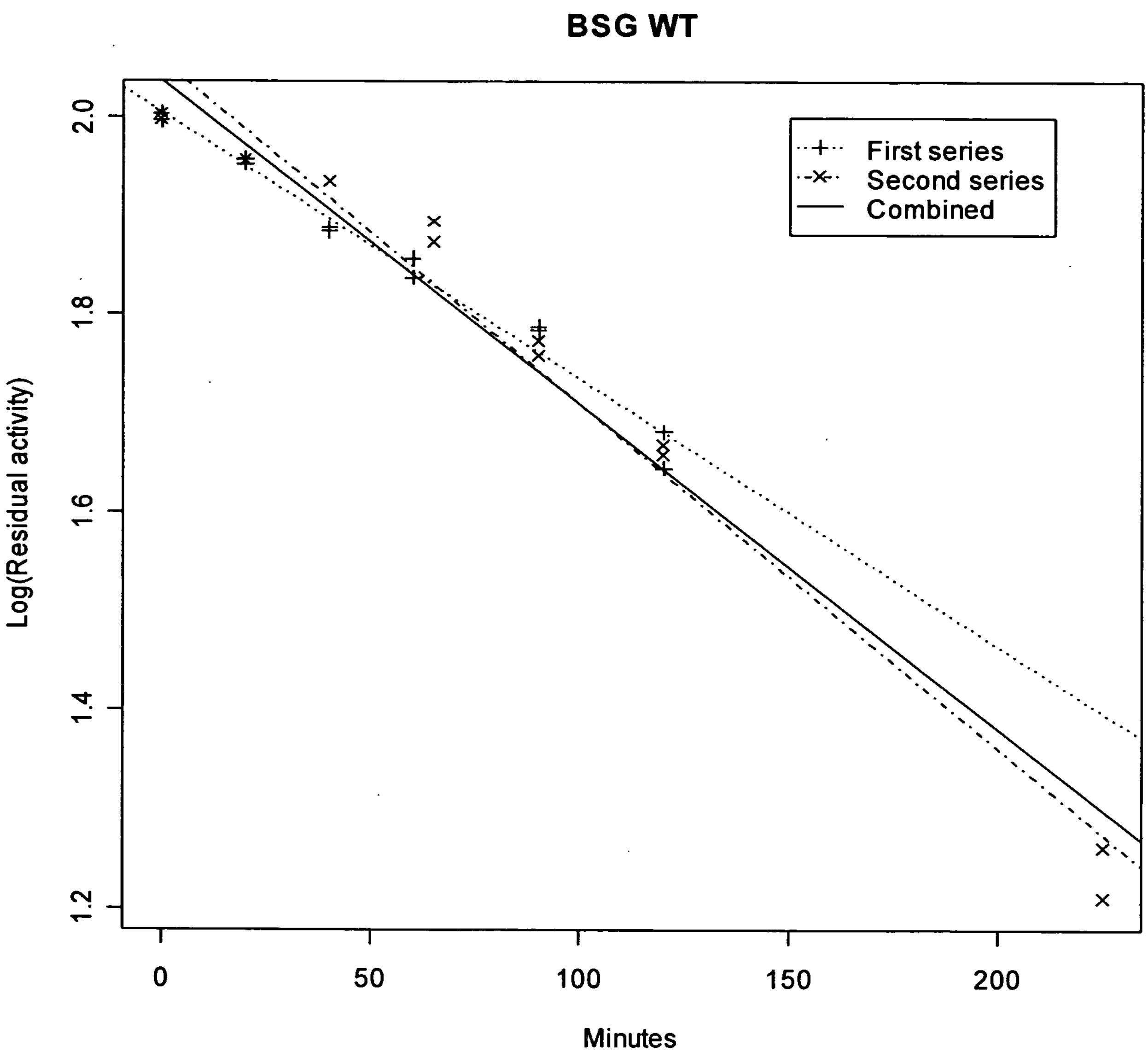
Date SEP 6th, 2004

Torben V. Borchert, Ph.D.

Appendix 1:

Differences between data-series, BSG:

BSG-WT

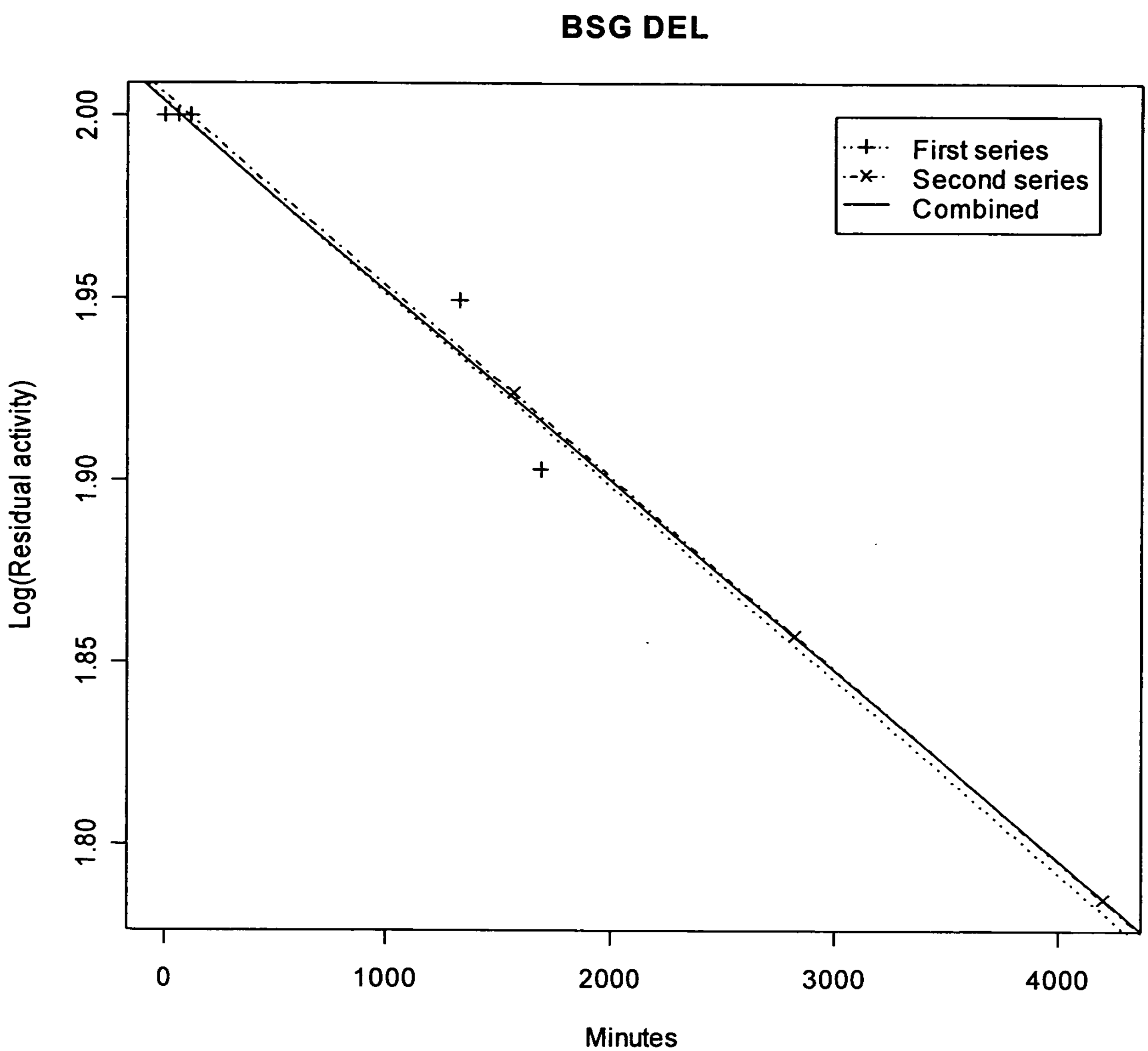


Summary of statistical analysis.

Below is a screen-dump from the statistical analysis, showing that there is a significant difference in the slope in the two data series. The p-value for same slope is underlined.
The analysis was done in R version 1.8.1 (http://www.r-project.org). The data for the BSG WT is held in the data-frame `bsg.wt` as shown in the table above. In the data-frame the time is called `var1`, the residual activity is `var2` and `var3` is a factor over the two series of experiments. The output shows the effect of the factor on a linear regression on the Log(residual activity) over incubation time.

```
> summary(lm(log10(bsg.wt$var2)~bsg.wt$var1*bsg.wt$var3))

Call:
lm(formula = log10(bsg.wt$var2) ~ bsg.wt$var1 * bsg.wt$var3)

Residuals:
      Min        1Q    Median        3Q       Max
-0.063233 -0.012558  0.001456  0.020149  0.063980

Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)              2.0043563  0.0165332 121.233  < 2e-16 ***
bsg.wt$var1             -0.0027017  0.0002416 -11.183 4.68e-10 ***
bsg.wt$var32             0.0520051  0.0226679   2.294   0.0327 *
bsg.wt$var1:bsg.wt$var32 -0.0007776  0.0002771  -2.806   0.0109 *
---
Signif. codes:  0 `***' 0.001 `**' 0.01 `*' 0.05 `.' 0.1 ` ' 1

Residual standard error: 0.03408 on 20 degrees of freedom
Multiple R-Squared: 0.9768,     Adjusted R-squared: 0.9733
F-statistic: 280.4 on 3 and 20 DF,  p-value: < 2.2e-16
```

BSG-DEL



Summary of statistical analysis.

Below is a screen-dump from the statistical analysis, showing that there is not a significant difference in the slope in the two data series. The p-value for same slope is underlined.
The analysis was done in R version 1.8.1 (http://www.r-project.org). The data for the BSG deletion is held in the data-frame `bsg.del` as shown in the table above. In the data-frame the time is called `var1`, the residual activity

is var2 and var3 is a factor over the two series of experiments. The output shows the effect of the factor on a linear regression on the Log(residual activity) over incubation time.

```
> summary(lm(log10(bsg.del$var2)~bsg.del$var1*bsg.del$var3))

Call:
lm(formula = log10(bsg.del$var2) ~ bsg.del$var1 * bsg.del$var3)

Residuals:
        1          2          3          4          5          6          7          8
-0.0043196 -0.0008682  0.0020522  0.0151601  0.0002194 -0.0120245 -0.0004197
0.0002003

Coefficients:
                           Estimate Std. Error t value Pr(>|t|)
(Intercept)                 2.004e+00  5.978e-03 335.303 4.75e-10 ***
bsg.del$var1               -5.310e-05  6.243e-06  -8.505  0.00105 **
bsg.del$var34               1.836e-03  1.739e-02   0.106  0.92103
bsg.del$var1:bsg.del$var34  4.731e-07  8.218e-06   0.058  0.95685
---
Signif. codes:  0 `***' 0.001 `**' 0.01 `*' 0.05 `.' 0.1 ` ' 1

Residual standard error: 0.009979 on 4 degrees of freedom
Multiple R-Squared: 0.9905,     Adjusted R-squared: 0.9834
F-statistic: 139.2 on 3 and 4 DF,  p-value: 0.0001682
```

Comparing thermo stabilization.

Analysis of significance of different stabilization:

We have the following slopes on the curves:

	Slope	Std err	Relative std err
BAN WT	-0.346932	0.042031	0.121152
BAN DEL	-0.031550	0.001153	0.036556
BSG WT	-0.003286	0.000128	0.039024
BSG DEL	-0.000052	0.000002	0.040217

We can compute the ratios of the slopes (which are the reciprocals of the ratios of the half-lifes).

	Ratio	Std err	Relative Std err
BAN WT / BAN DEL	10.9962346	1.3915416	0.1265471
BSG WT / BSG DEL	63.04430515	3.53290004	0.05603837

This means we have a ratio between the slopes of

$$\frac{63.0}{11.0} = 5.73$$

with a relative standard error of

$$\sqrt{0.127^2 + 0.056^2} = 0.138$$

and a standard error of

$$5.73 * 0.138 = 0.79$$

So, if we use the golden rule of standard errors, that the true value is within +/- two standard errors of the estimated value, we have that **the deletion has a stabilizing effect in BSG which is between 4 and 7 times what is seen in BAN**.

C.V.

Torben V. Borchert
Biskop Svanes Vej 65A, 1th
DK-3460 Birkerød
Denmark

Protein Design, Novozymes
Building 1U1.23
DK-2880 Bagsværd,
Denmark
Tel. +45 44 42 69 77
Fax. +45 44 98 02 46
Mob. +45 23 71 31 48

EDUCATION:

March 1991	Ph.D. The technical University of Denmark (DTH) DK-2800 Lyngby Denmark.
February 1988	M.S. in biochemical engineering (civilingenior) The technical University of Denmark (DTH) DK-2800 Lyngby Denmark.

ADDITIONAL EDUCATION/QUALIFICATIONS:

2003	Nz leadership competences
Fall 2001	DIEU: Assertionstræning
1996	Project Management
Fall 1994	Course on Communication Engineering and Business Administration (EBA) Ingeniorhojskolen, Kobenhavns Teknikum.
June 1994	Course on Marketing Engineering and Business Administration (EBA) Ingeniorhojskolen, Kobenhavns Teknikum.
April 1992	Cold Spring Harbor Laboratory course on

"Protein purification and characterization"

PAST & CURRENT APPOINTMENTS:

Sep 1993- Present	Director, Protein Design Senior Manager, Protein Design Principal Scientist Chemist (research scientist) Novozymes (Novo Nordisk) Molecular Biotechnology Bagsvaerd, Denmark.
May 1991- Aug. 1993	Post doctoral fellow European Molecular Biology Laboratory D-6900 Heidelberg Germany.
Jan. 1991- Apr. 1991.	Worked on a project for Valio, Finnish Co-operative dairies association, Research and Development Centre. P.O. Box 176, SF 00181 Helsinki, Finland. This work was carried out at The technical University of Denmark.
Nov. 1989- Dec. 1990	Graduate student The technical University of Denmark. Dept. of Microbiologi. DK-2800 Lyngby, Denmark.
Apr. 1988- Oct. 1989	Worked as "Visiting Scientist" at E.I. du Pont de Nemours & Co., Experimental Station, Wilmington, Delaware 19880, USA.
Feb. 1987-	M.S. project.

Feb.1988 Dept. of Microbiology, The technical University of Denmark.

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Editor:

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<u>Issued Patents:</u>

US 5,753,460 (amylase variants)
US 5,801,043 (amylase variants)
US 5,830,837 (amylase variants)
US 5,989,169 (amylase variants)
US 6,022,724 (amylase mutants)
US 6,093,562 (amylase variants)
US 6,143,708 (amylase mutants)
US 6,159,687 (method for generating recombined polynucleotides)
US 6,159,688 (method of producing polynucleotide variants)
US 6,165,718 (method for in vivo production of a mutant library in cells)
US 6,187,576 ((amylase variants)
US 6,204,232 (amylase mutants)
US 6,291,165 (shuffling of heterologous DNA sequences)
US 6,297,038 (amylase variants)
US 6,309,871 (alkaline amylases)
US 6,326,206 (in vivo recombination)
US 6,361,989 (amylases)
US 6,368,805 (directed recombination)
US 6,436,888 (amylases)
US 6,440,716 (amylases)
US 6,518,042 (diversity generation)
US 6,528,298 (amylases)
US 6,541,207 (recombination method)